Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

Antibiotic Resistance of *Escherichia coli* in Free-Ranging Yaks (*Bos grunniens*) from Tibetan Plateau, China

Mujeeb Ur Rehman[1], Hui Zhang[1], Muhammad Kashif Iqbal[1], Fazul Nabi[1], Shucheng Huang[1], Yanfang Lan[1], Khalid Mehmood[1,3], Houqiang Luo[1] and Jiakui Li[1,2*]

[1]College of Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, P.R. China; [2]Laboratory of Detection and Monitoring of Highland Animal Diseases, Tibet Agricultural and Animal Husbandry College, Linzhi, 860000, Tibet, P.R. China; [3]College of Veterinary and Animal Sciences, Islamia University of Bahawalpur, Pakistan
*Corresponding author: lijk210@sina.com

ARTICLE HISTORY (16-290)

Received: November 9, 2016
Revised: March 3, 2017
Accepted: March 4, 2017
Published online: March 18, 2017

Key words:
Antimicrobial-resistant
Disk diffusion method
Double-disk synergy method
Escherichia coli

ABSTRACT

In this study, we collected 565 fecal samples from free-range Tibetan yaks to examine whether these animals can serve as a reservoir for pathogenic and antimicrobial-resistant *Escherichia coli*. The antimicrobial sensitivity testing of each resulting isolate was evaluated according to the disk diffusion method, whereas PCR analysis was performed for detection of resistance and virulence genes. Additionally, strains containing extended-spectrum beta-lactamases (ESBLs) genes were initially identified via the double-disk synergy method and confirmed by PCR analysis. Of the 488 *E. coli* isolates examined, approximately 31.1% were multidrug resistant. *OmpA* (30.2%) and *etrA* (23.1%), and bla_{CTX-M} (27.6%) and bla_{TEM} (14.4%) were the mainly prevalent virulence and drug resistance genes, respectively. Notably, we detected the CTX-M type but not the SHV type among the ESBL-producing strains and there was a significant association between resistance and virulence genes for *aac(3)-lla*, bla_{TEM}/*ompA*, bla_{CTX-M}/*etrA*, and *cmlA*/*cnf1* ($P<0.05$). Lastly, the majority of the strains belonged to phylogroup A (72.7%). This is the first report of the occurrence of pathogenic and antimicrobial-resistant ESBL-producing *E. coli* strains in free-range Tibetan yaks.

To Cite This Article: Rehman MU, Zhang H, Iqbal MK, Nabi F, Huang S, Lan Y, Mehmood K, Luo H and Li J, 2017. Antibiotic resistance of *Escherichia coli* in free-ranging yaks (*Bos grunniens*) from Tibetan Plateau, China. Pak Vet J, 37(2): 139-144.

INTRODUCTION

Escherichia coli (*E. coli*) is an essential and commensal bacterium that is generally located as a part of the microflora of the human and animal intestinal tract (Kaper *et al.*, 2004). Although most *E. coli* strains are considered benign, some have been shown to cause colibacillosis, septicemia, enterotoxaemia, and calf dysentery in yaks, which are associated with mortality rates of up to 23% (Hongning *et al*., 2004; Naganandhini *et al*., 2015). Currently, *E. coli* infections are treated with a variety of antibiotics such as penicillin, streptomycin, and gentamicin, which has resulted in increases in both the production cost and the rates of microbial resistance to these compounds. Notably, antimicrobial susceptibility testing and bacterial culturing are not always carried out for directing antibiotic selection, leading to improper practical treatment. In particular, the emergence of antimicrobial-resistant strains of *E. coli* has become a serious problem in veterinary medicine, and has resulted in there being a limited number of therapeutic options available to practitioners (Hongning *et al*., 2004). As such, it is essential to present a scientific foundation for the rational and secure use of antibiotics. Furthermore, the widespread utilization of cephalosporins to ensure successful antimicrobial therapy has led to the persistent emergence of strains that encode novel extended spectrum beta-lactamases (ESBLs), which are the major cause of sensitivity to cephalosporins and penicillin. Indeed, more than 300 types of ESBLs have been characterized to date (Zhang *et al*., 2010). As a result, multiple researchers are currently attempting to reduce the frequency of administration of extended-spectrum cephalosporins to thereby prevent the emergence of drug resistant bacteria.

Antibiotic resistance is a natural phenomenon. Data regarding the prevalence of antibiotic resistance and the types of ESBLs produced by *E. coli* are available for certain regions of China and from many other countries

(Zhang *et al*., 2010; Naganandhini *et al*., 2015; Yu-xi *et al*., 2015; Zhang *et al*., 2017). However, despite the relevance of the virulence potential, antimicrobial resistance, and ESBL-production of *E. coli* strains to agriculture and human health, there have been few studies to examine the *E. coli* strains present in free ranging animals. We therefore chose to isolate *E. coli* strains from the feces of free-ranging Tibetan yaks that grazed on a semi-mountainous area and which had not been exposed to antimicrobials for growth promotion or as therapeutic agents. The virulence potential and antibiotic resistance profiles of these strains were then evaluated to find out the potential impact of free-range Tibetan yaks as reservoirs for antimicrobial-resistant *E. coli*.

MATERIALS AND METHODS

Sample collection, isolation and identification: In this study, 565 rectal swabs were randomly collected from adult free-range yaks in the Nyingchi (n=412 from Bayi, Bujiu, Mirui, Lilong, Nanyi, Cuogao, Bahe, and Qiangna) and Naqu (n=153 from Naqu, Anduo, and Biru) regions of Tibet between June 2015 and February 2016. A single fecal sample was collected from each yak during sampling, and only one *E. coli* isolate was examined per sample. Samples were gathered near the animals, placed in bags, and stored at 4°C. The samples were then transported to Huazhong Agricultural University, Wuhan for additional experiments. Nutrient broth and MacConkey agar (all agars purchased from Hangzhou, Reagents, Co., Ltd., China) were used to enrich and streak the samples, respectively. Pink-colored colonies on MacConkey agar were subsequently used to inoculate eosin methylene blue agar (EMB), and greenish metallic-colored colonies on EMB were considered *E. coli*. Strains were then confirmed as *E. coli* via Gram staining and biochemical analysis, using the API 20E system (BioMerieux, Marcy-l Etoile, France). Confirmed strains were stored at -80°C in 20% glycerol.

Antibiotic sensitivity testing and screening of ESBL-producing strains: The antibiotic sensitivity profile for each isolate was evaluated according to the disk diffusion test, per criteria suggested by the CLSI (Clinical and Laboratory Standards Institute, 2014) by using Mueller-Hinton agar as the test medium for each of the following classes of antibiotics (all purchased from GE Hangwei Medical Systems Co., Ltd., Beijing, China): ampicillin (10 µg), ceftriaxone (30 µg), ceftazidime (30 µg), chloramphenicol (30 µg), gentamicin (10 µg), ofloxacin (5 µg), streptomycin (10 µg), sulphonamide (300 µg), tetracycline (30 µg), and trimethoprim/sulfamethoxazole (1.25/23.75 µg). Strains exhibiting resistance to at least one beta lactam were subjected to ESBL phenotypic conformation via the double disk synergy test, using cefotaxime (CTX, 30 µg), and ceftriaxone (CRO, 30 µg) and ceftazidime (CAZ, 30 µg) disks, per CLSI (2014) criteria. Isolates showing simultaneous resistance to three or more different classes of antibiotics were considered multidrug resistant (MDR). Strains *E. coli* ATCC 25922 and *E. coli* ATCC 35218 were used as positive and negative controls, respectively.

Screening for resistance genes and virulence-associated genes: DNA of *Escherichia coli* strains was extracted via boiling, as previously described (Yu-xi *et al*., 2015), and used as DNA template for PCR reactions to reveal resistance genes and virulence-associated genes by using previously described primers and methodology, but with suitable modifications (Table 1). Amplification products were then separated and quantified via 1% agarose gel electrophoresis. Resulting bands were observed using a Gene Genius BioImaging System, (SynGene, Cambridge, UK), and band sizes were determined by comparing to a DNA marker (Tiangen Biotec, Beijing, China).

All the PCR products were purified and sequenced by Wuhan Qingke Biotechnology CO., Ltd. (Wuhan, China). The resulting nucleotide segments and deduced amino acid sequences were compared with those contained in NCBI GenBank and at http://www.lahey.org/studies/ for the identification of specific types of ESBL genes.

Phylo groups of *Escherichia coli:* *Escherichia coli* strains were allocated to phylogenetic groups A, B1, B2, or D following triplex PCR analysis using a previously described protocol and primers (Clermont *et al*., 2000), but with suitable modifications for targeting *chuA*, *yjaA*, and the DNA fragment TspE4.C2. The results were interpreted accordingly as suggested by (Clermont *et al*., 2000).

Statistical analysis: Variables are expressed as percentages (%). $P<0.05$ of Chi-squared test were regarded as significant. All analyses were conducted using Stata 11 software (Stata Corp LP, College Station, TX, USA).

RESULTS

Isolation rate of *Escherichia coli:* Overall, 488 *E. coli* strains were recovered from the 565 fecal samples examined, yielding an isolation rate of 86.4%, based on biochemical analyses.

Antibiotic resistance patterns of *Escherichia coli* isolates: All strains were grouped on the basis of phenotypic resistance (Table 2). Resistance to no less than three different classes of antibiotics was observed in 31.1% of the *E. coli* isolates; these strains were therefore considered MDR. Among the 488 *E. coli* isolates tested, we observed high rates of sensitivity to ofloxacin (99.6%), ceftazidime (98.0%), chloramphenicol (97.3%), and streptomycin (96.7%) and moderate rates of sensitivity to sulphonamide (96.1%) and trimethoprim/sulfametho-xazole (96.3%). Resistance to tetracycline (7.8%), ampicillin (6.1%), ceftriaxone (4.5%), and gentamicin (4.1%) was prevalent among the *E. coli* strains tested; however, only 2% and 0.4% of strains were resistant to ceftazidime and ofloxacin, respectively.

Detection of ESBL genes: Thirty-two ESBL-producing *Escherichia coli* strains were identified via the combination of DDST and PCR analyses, resulting in an overall prevalence of 6.5%. Subsequent sequencing analyses indicated that these 32 isolates were comprised of 21 $bla_{CTX\text{-}M\text{-}15}$ strains, six $bla_{CTX\text{-}M\text{-}14}$ strains, and five $bla_{CTX\text{-}M\text{-}1}$ strains. Notably, each of these 32 isolates was positive for the *blaCTX-M* genes but negative for the *blaSHV* and other beta lactamase genes tested (Fig. 1).

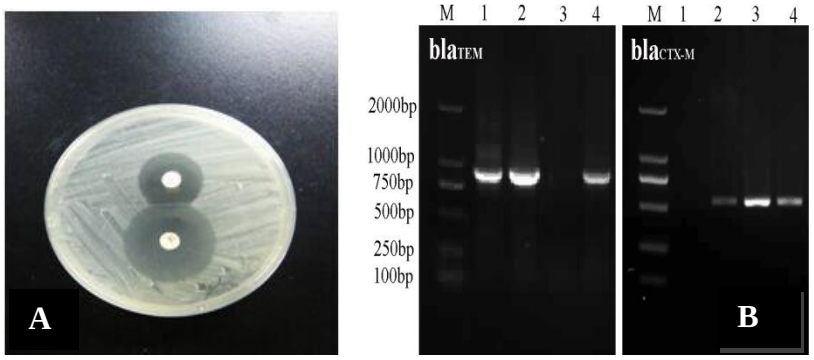

Fig. 1: Phenotypic (A) and genotypic (B) characterization of the beta-lactamases-producing *Escherichia coli* strains (bla_{CTX-M} and bla_{TEM}: where, M= Marker and Line 1, 2, 3, 4 are positive and negative isolates) by using double disk synergy test and PCR analysis, respectively.

Table 1: PCR primers used in this study

Target Genes	Primer Sequence (5′–3′)	Product size (bp)	Reference
Shiga toxin (*stx*1)	ATTCGCTGAATGTCATTCGCT ACGCTTCCCAGAATTGCATTA	664	Cheng *et al.* (2006)
Shiga toxin (*stx*2)	GAATGAAGAAGATGTTTATAGCGG GGTTATGCCTCAGTCATTATTAA	281	Cheng *et al.* (2006)
Intimin (*eae*A)	ATATCCGTTTTAATGGCTATCT AATCTTCTGCGTACTGTGTTCA	425	Cheng *et al.* (2006)
Outer membrane protein (*omp*A)	AGCTATCGCGATTGCAGTG GGTGTTGCCAGTAACCGG	919	Yu-xi *et al.* (2015)
Aerobatin (*aer*)	TACCGGATTGTCATATGCAGACCGT AATATCTTCCTCCAGTCCGGAGAAG	602	Alabsi *et al.* (2014)
Cytotoxic necrotizing factor 1 (*cnf*1)	AAGATGGAGTTTCCTATGCAGGAG CATTCAGAGTCCTGCCCTCATTATT	498	Alabsi *et al.* (2014)
Enterohemolysin (*exhA*)	GCATCATCAAGCGTACGTTCC AATGAGCCAAGCTGGTTAAGCT	534	Chapman *et al.* (2006)
α-hemolysin (*hlyA*)	AACAAGGATAAGCACTGTTCTGGCT ACCATATAAGCGGTCATTCCCGTCA	1177	Chapman *et al.* (2006)
O4 lipopolysaccharide synthesis (*rfc*)	ATCCATCAGGAGGGGACTGGA AACCATACCAACCAATGCGAG	788	Chapman *et al.* (2006)
Serum survival protein (*iss*)	CAGCAACCCGAACCACTTGATG AGCATTGCCAGAGCGGCAGAA	323	Chapman *et al.* (2006)
F4 fimbrial adhesion (*faeG*)	GGTGATTTCAATGGTTCG ATTGCTACGTTCAGCGGAGCG	764	Chapman *et al.* (2006)
Component of ETT2 type III secretion system (*etr*A)	CTTCTTCCTAACGAAACTATCATTA TGACATATCAACTTTCTCTTACGC	913	Hartleib *et al.* (2003)
Beta lactam (*blaTEM*)	GAGTATTCAACATTTCCGTGTCGC TACCAATGCTTAATCAGTGAGGC	860	Zhang *et al.* (2010)
Beta lactam (*blaSHV*)	GGG TTA TTC TTA TTT GTC GC TTA GCG TTG CCA GTG CTC	567	Chen *et al.* (2010)
Beta lactam (*blaCTX-M*)	ATGTGCAGYACCAGTAARGTKATGGC TGGGTRAARTARGTSACCAGAAYCAGCGG	593	Hasman *et al.* (2005)
Amino glycosides (*aac(3)-lla*)	ACCCTACGAGGAGACTCTGAATG CCAAGCATCGGCATCTCATA	384	Guo *et al.* (2015)
Amino glycosides (*aac(6')-lb*)	ATGACCTTGCGATGCTCTATG CGAATGCCTGGCGTGTTT	486	Guo *et al.* (2015)
amino glycosides (*aph(3')-lla*)	TGACTGGGCACAACAGACAA CGGCGATACCGTAAAGCAC	677	Guo *et al.* (2015)
Amino glycosides (*ant(3')-la*)	ATCTGGCTATCTTGCTGACA TATGACGGGCTGATACTGG	284	Guo *et al.* (2015)
Efflux protein (*floR*)	GGCTTTCGTCATTGCGTCTC ATCGGTAGGATGAAGGTGAGGA	650	Zhang *et al.* (2009)
Efflux protein (*cmlA*)	TGCCAGCAGTGCCGTTTAT CACCGCCCAAGCAGAAGTA	900	Zhang *et al.* (2009)
Tetracycline (*tetA*)	GGCACCGAATGCGTATGAT AAGCGAGCGGGTTGAGAG	480	Guo *et al.* (2015)
Tetracycline (*tetC*)	CTCAGTATTCCAAGCCTTTC CTAAGCACTTGTCTCCTGTT	416	Guo *et al.* (2015)
Tetracycline (*tetM*)	CTGGGCTGCTTCCTAATGC AGCTGTCCCTGATGGTCGT	580	Guo *et al.* (2015)
Dihydropteroate synthetase (*sul1*)	CGGCGTGGGCTACCTGAACG GCCGATCGCGTGAAGTTCCG	433	Zhang *et al.* (2009)
Dihydropteroate synthetase (*sul2*)	GCGCTCAAGGCAGATGGCATT GCGTTTGATACCGGCACCCGT	293	Zhang *et al.* (2009)
Dihydropteroate synthetase (*sul3*)	AGATGTGATTGATTTGGGAGC TAGTTGTTTCTGGATTAGAGCCT	443	Zhang *et al.* (2009)
Trimethoprim (*dfrA1*)	GTGAAACTATCACTAATGG ACCCTTTTGCCAGATTTG	471	Seputiene *et al.* (2010)
Trimethoprim (*dfrA17*)	AAAATTTCATTGATTTCTGCA TTAGCCTTTTTTCCAAATCT	471	Seputiene *et al.* (2010)

Table 2: Distribution of antibiotic resistance, phylogroups, and antimicrobial resistance genes detected in *Escherichia coli* strains isolated from free-range Tibetan yaks

Antimicrobial agents (n)	Resistance gene (s)	No. of positive isolates (%) n=116	Phylogenetic group of resistant gene (%)			
			A (n=355)	B1 (n=45)	B2 (n=56)	D (n=32)
Amino glycoside (33)	*aac(3)-lla*	17 (14.7)	7 (2)	2 (4.4)	2 (3.6)	6 (18.7)
	ant(3')-la	9 (7.7)	3 (0.8)	4 (8.9)	1 (1.8)	1 (3.1)
	aac(3)-lla+ ant(3')-la	1 (0.9)	0	1 (2.2)	0	0
	aph(3')-lla	0	–	–	–	–
	aac(6')-lb	0	–	–	–	–
Beta-lactam (62)	bla_{TEM}	19 (16.4)	5 (1.4)	7 (15.5)	4 (7.1)	3 (9.4)
	bla_{CTX-M}	32 (27.6)	16 (4.5)	5 (11.1)	8 (14.3)	3 (9.4)
	bla_{SHV}	0	–	–	–	–
	bla_{TEM} + bla_{CTX-M}	0	–	–	–	–
Chloramphenicol (13)	*floR*	0	–	–	–	–
	cmlA	6 (5.2)	3 (0.8)	1 (0.3)	0	2 (6.2)
Quinolone (2)	–	–	–	–	–	–
Tetracycline (38)	*tetA* and *tetC*	0	–	–	–	–
	tetM	16 (13.8)	11 (3.1)	0	1 (1.8)	4 (12.5)
Trimethoprim (15)	*dfrA1*	0	–	–	–	–
	dfrA17	9 (7.7)	3 (0.8)	3 (6.7)	2 (3.6)	1 (3.1)
Sulfonamide (19)	*sul1*	7 (6)	7 (2)	0	0	0
	sul2 and *Sul3*	0	–	–	–	–

- indicates no results available or not determined

Table 3: Distribution and combination patterns of drug resistance and virulence genes among *Escherichia coli* strains isolated in this study

Virulence gene (s)	No. of isolates (%) n=169	Phylogenetic group (%)			
		A (n=355)	B1 (n=45)	B2 (n=56)	D (n=32)
ompA	51 (30.2)	36 (10.1)	3 (6.7)	7 (12.5)	5 (15.6)
etrA	39 (23.1)	21 (6)	5 (11.1)	4 (7.1)	9 (28.1)
aer	33 (19.5)	19 (5.3)	4 (8.9)	7 (1.8)	3 (9.4)
cnf1	22 (13)	13 (3.7)	3 (6.7)	2 (3.6)	4 (12.5)
hlyA	14 (8.2)	5 (1.4)	2 (4.4)	4 (7.1)	3 (9.4)
C1	3 (1.8)	3 (0.8)	0	0	0
C2	4 (2.4)	3 (0.8)	0	1 (1.8)	0
C3	2 (1.2)	0	1 (2.2)	1 (1.8)	0
C4	1 (0.6)	0	0	1 (1.8)	0

Key: C1, combination of *ompA* + *aer* + *etrA* + *hlyA* genes; C2, combination of *ompA* + *aer* genes; C3, combination of *etrA* + *cnf1* genes; C4, combination of *ompA* + *aer* + *etrA* + *cnf1* genes.

Detection of resistance and virulence genes: The most prevalent resistance genes detected were those encoding resistance to beta lactams [bla_{CTM-X} (27.6%) and bla_{TEM} (16.4%)], amino glycosides [*aac(3)-lla* (14.7%) and *ant(3')-la*, (7.7%)], tetracycline (*tetM*, 13.8%), chloramphenicol (*cmlA*, 5.2%), trimethoprim (*dfrA17*, 7.7%) and sulfonamide (*sul1*, 6%). Fifty-one isolates (10.4%) carried at least one gene encoding similar resistance phenotypes. Meanwhile, several yaks harbored strains encoding a multigene locus containing *aac(3)-lla* and *ant(3')-la* [*aac(3)-lla*/*ant(3')-la*] (0.9%). Notably, approximately 60% of the phenotypic resistant isolates harbored none of the resistance-encoding genes tested.

Of the 488 *E. coli* isolates tested, 159 (32.6%) were positive for at least one of the virulence gene tested. Of these, 30.2% were positive for *omp*A, while 23.1%, 19.5%, 13.0%, and 8.3% were positive for *etr*A, *aer*, *cnf*1, and *hlyA*, respectively. Conversely, all isolates were negative for the other genes tested. Notably, 10 isolates were positive for two or more virulence genes: *omp*A+*aer*+*etr*A+*hylA* (3 isolates); *omp*A+*aer* (4 isolates); *etr*A+*cnf*1 (2 isolates); and *omp*A+*aer*+*etr*A+*cnf*1 (1 isolate).

Occurrence of phylogroups: Most of the *E. coli* isolates from free-range Tibetan yaks belonged to phylogroup A (72.7%), with the other isolates belonging to phylogroup B1 (9.2%), B2 (11.5%) and D (6.6%). Table 2 and Table 3 summarize the distributions of antibiotic resistance and virulence genes among the different phylogenetic groups examined.

Association between antimicrobial resistance and virulence genes: There was a statistically significant association ($P<0.05$) between the presence of the following antimicrobial resistance and virulence genes: *aac(3)-lla*, bla_{TEM}/*ompA*, bla_{CTX-M}/*etrA*, and *cmlA*/*cnf1*. Conversely, there were no significant links ($P<0.05$) among other resistance or virulence genes tested (Table 4).

DISCUSSION

We detected low carriage rates of virulence-associated genes, ESBL genes, and antimicrobial-resistance genes. The antimicrobial resistance patterns observed among the *E. coli* strains tested showed to link with the type of antibiotics utilized in veterinary medicine. Our findings indicate that these patterns were not due to phenotypic resistance, as has previously been observed in *E. coli* isolated from yaks or other animals in China (Hongning *et al*., 2004; Zhang *et al*., 2010; Liu *et al*., 2012), as almost all *E. coli* strains were sensitive to ofloxacin and chloramphenicol. Even if we did not assay for susceptibility to all classes of drugs (e.g., fluoroquinolones) that inhibit the development of *E. coli*, the majority of the strains were resistant to two or more classes of antibiotics. In particular, the highest observed rates of resistance were to tetracycline and ampicillin. Consistent with these findings, low levels of resistance to commonly used antibiotics were also detected in free-range animals in Spain (Navarro-Gonzalez *et al*., 2013). These findings are to be expected, as these antibiotics continue to be consumed as growth promoters, and for disease prevention and therapy, in yak farming in China. As such, antibiotic resistant strains could readily be spread via direct contact, as well as through cross contamination of water, food, and feces, between yaks and to other farm animals present within the study area. In addition, the overall prevalence of the resistance genes detected in our study was similar to the appurtenance of the phenotypes, indicating their usual expression. The co-existence of resistance genes encoding similar phenotypes may be explained by the acquisition of distinct genetic elements containing these genes.

Table 4: Associations between the presence of virulence genes and antimicrobial resistant genes in *Escherichia coli* strains isolated from free-range Tibetan yaks

Genes		*ompA*			*etrA*			*aer*			*cnf1*			*hylA*		
		Pos	Neg	P- value	Pos	Neg	P- value	Pos	Neg	P- value	Pos	Neg	P- value	Pos	Neg	P- value
aac(3)-lla	Pos	6	11	<0.001*	2	15	0.559	3	14	0.069	2	15	0.142	1	16	0.449
	Neg	45	426		37	434		30	441		20	451		13	458	
ant(3')-la	Pos	1	8	0.978	0	9	0.372	2	7	0.062	0	9	0.511	0	9	0.603
	Neg	50	429		39	440		31	448		22	457		14	465	
bla_{TEM}	Pos	7	12	<0.001*	3	16	0.201	1	18	0.791	2	17	0.197	1	18	0.524
	Neg	44	425		36	433		32	437		20	449		13	456	
bla_{CTX-M}	Pos	6	26	0.112	7	25	0.003*	4	28	0.181	3	29	0.170	0	32	0.315
	Neg	45	411		32	424		29	427		19	437		14	442	
cmlA	Pos	1	3	0.340	0	4	0.554	0	4	0.589	1	3	0.047*	0	4	0.730
	Neg	50	434		39	445		33	451		21	463		14	470	
TetM	Pos	3	10	0.132	2	11	0.319	3	10	0.018*	1	12	0.575	0	13	0.530
	Neg	48	427		37	438		30	445		21	454		14	461	
sul1	Pos	0	1	0.732	0	1	0.768	0	1	0.788	0	1	0.828	0	1	0.863
	Ne.	51	436		39	448		33	454		22	465		14	473	
dfrA17	Pos	2	7	0.244	1	8	0.728	0	9	0.415	1	8	0.335	0	9	0.603
	Ne.	49	430		38	441		33	446		21	458		14	465	

Pos: no. of positive genes; Neg: no. of negative genes; *P*- value for significant associations between genes. *Significant association (P<0.05) between antibiotic resistant and virulence genes.

The virulence genes investigated in this study were chosen based on their significance to virulence in both animals and humans. The high prevalence of the *ompA* and *etrA* genes was consistent with previous findings obtained from non-pathogenic *E. coli* strains (Hartleib *et al*., 2003). Contrary to our results, however, a previous study detected a high prevalence of both *stx1* and *stx2* in Qinghai Tibetan yaks (Bai *et al*., 2013). In this study, with the exception of the few genes pairs, there were no statistical associations among the occurrence of virulence and resistance genes to the antibiotics tested, despite the presence of such genes. These results indicate that there are other factors also associated with resistance to commonly used antibiotics.

It was previously reported that extra-intestinal pathogenic *E. coli* (ExPEC), which is a prominent zoonotic infection that is responsible for various illnesses, particularly urinary tract infections in humans and animals, is mainly caused by strains belonging to phylogenetic groups B2 and D (Nandanwar *et al*., 2014). In contrast, groups A and B1 were reported to be avirulent and more common among animals than humans (Huber *et al*., 2013). The prevalence of virulence genes identified was therefore inconsistent with the appurtenance of the strains examined in this study.

We detected ESBL genes among various yak *E. coli* isolates. Similar results were recorded in a previous study of fowl and pigs in Henan, China (Zhang *et al*., 2010). Moreover, another researcher observed the increases among the rate of ESBLs in *E. coli* strains isolated from cattle and pigs (Geser *et al*., 2011). Here, we detected CTX-M-derived ESBLs belonging to the CTX-M-1 (CTX-M-15 and CTX-M-1) and CTX-M-9 (CTX-M-14) groups, which were earlier reported to be linked with ExPEC strains and were highlighted as one of the prominent zoonotic risks related to antimicrobial resistance in *E. coli* from animals. Indeed, free-range animals have been found to function as carriers of ESBL-producing *E. coli* isolates in different countries, including Portugal (Semedo-Lemsaddek *et al*., 2013) and Bangladesh (Hasan *et al*., 2012). Regardless of the usually low incidence of ESBLs detected, these data show that sheltered natural atmospheres are not free from the beginning of anthropogenic antibiotic resistance.

Conclusions: Free ranging yaks might play active role as reservoirs and transmitters of threatening pathogens than previously thought. A number of reasons are likely to play role in the wide-range distribution of antimicrobial or virulence genes, together with the frequent use of natural resources and close contact with other animals, combined with unlimited use of antimicrobial agents in veterinary medicine. Thus, the development of a strong scrutiny system and other appraises are required to control the spread of these resistant strains among livestock in Tibet and other nomadic pastoral locations.

Acknowledgments: This study was supported by the Tibet Autonomous Region Science Fund and the Chinese Agricultural Research Systems (CARS-38); Agriculture and Animal Husbandry Research Collaborative Innovation Project: Yak important epidemiological disease investigation and prevention research by the Agricultural and Animal Husbandry College of Tibet University.

Authors contribution: JKL and MUR conceived and designed the experiments; MUR and HZ performed the experiments; MKI, KM, FN, HCS, YL, and HL contributed in sampling, writing, and analysis tools; MUR and JKL wrote the manuscript.

REFERENCES

Alabsi MS, Ghazal A, Sabry SA, *et al.,* 2014. Association of some virulence genes with antibiotic resistance among uropathogenic *Escherichia coli* isolated from urinary tract infection patients in Alexandria, Egypt: A hospital-based study. J Global Antimicro Res 2:83-6.

Bai X, Zhao A, Lan R, *et al.*, 2013. Shiga toxin producing *Escherichia coli* in yaks (*Bos grunniens*) from the Qinghai Tibetan Plateau, China. PloS ONE 8:e65537.

Chapman TA, Wu XY, Barchia I, *et al.*, 2006. Comparison of virulence gene profiles of *Escherichia coli* strains isolated from healthy and diarrheic swine. Appl Environ Microbiol 72:4782-95.

Chen H, Shu W, Chang X, *et al.*, 2010. The profile of antibiotics resistance and integrons of extended-spectrum beta-lactamase producing thermo tolerant coliforms isolated from the Yangtze River basin in Chongqing. Environ Pollution 158:2459-64.

Cheng D, Sun H, Xu J and Gao S, 2006. PCR detection of virulence factors genes in *Escherichia coli* isolates from weaned piglets with edema disease and/or diarrhea in China. J Vet Microbiol 115:320-8.

Clermont O, Bonacorsi S and Bingen E, 2000. Rapid and simple determination of the *Escherichia coli* phylogenetic group. Appl Environ Microbiol 66:4555-8.

CLSI, 2014. Performance standards for antimicrobial susceptibility testing; twenty-first informational supplement. CLSI/NCCLS M100-S24. Clinical and Laboratory Standards Institute, Wayne, PA.

Geser N, Stephan R, Kuhnert P *et al.*, 2011. Fecal carriage of extended spectrum beta lactamase producing Enterobacteriaceae in swine and cattle at slaughter in Switzerland. J Food Protect 74:446-9.

Guo L, Long M, Huang Y, *et al.,* 2015. Antimicrobial and disinfectant resistance of *Escherichia coli* isolated from giant pandas. J Appl Microbiol 119:55-64.

Hartleib S, Prager R, Hedenstrom I, *et al.,* 2003. Prevalence of the new, SPI1-like, pathogenicity island ETT2 among *Escherichia coli.* Int J Med Microbiol 292:487-93.

Hasan B, Sandegren L, Melhus A, *et al.*, 2012. Antimicrobial drug–resistant *Escherichia coli* in wild birds and free-range poultry, Bangladesh. Emerg Infect Dis 18:2055-8.

Hasman H, Mevius D, Veldman K, *et al.,* 2005. Beta-Lactamases among extended-spectrum beta-lactamase (ESBL)-resistant Salmonella from poultry, poultry products and human patients in the Netherlands. J Antimicrob Chemother 56:115-21.

Hongning W, Juan L, Lang T, *et al.,* 2004. The mensuration of drug resistance of yaks *E. coli.* Proceedings of the fourth International Congress on yak 2004; Jincheng Z, Xiangdong Z, Jianlin H and Zhihua C (eds), Chengdu, Sichuan China, 19-sep, 2014. www.ivis.org/proceedings/yaks/2004/session5/Hongning.pdf

Huber H, Zweilfel C, Wittenbrink MM, *et al*., 2013. ESBL-Producing uropathogenic *Escherichia coli* isolated from dogs and cats in Switzerland. Vet Microbiol 162:992-6.

Kaper JB, Nataro JP and Mobley HL, 2004. Pathogenic *Escherichia coli*. Nat Rev Microbiol 2:123-40.

Liu Y, Liu K, Lai J, *et al*., 2012. Prevalence and antimicrobial resistance of Enterococcus species of food animal origin from Beijing and Shandong province, China. J Appl Microbiol 114:555-63.

Naganandhini S, Kennedy ZJ, Uyttendaele M, *et al*., 2015. Persistence of pathogenic and non-pathogenic *Escherichia coli* strains in various tropical agricultural soils of India. PLoS ONE 10:e0130038.

Nandanwar N, Janssen T, Kühl M, *et al.,* 2014. Extra intestinal pathogenic *Escherichia coli* (ExPEC) of human and avian origin belonging to sequence type complex 95 (STC95) portray indistinguishable virulence features. Int J Med Microbiol 304:835-42.

Navarro-Gonzalez N, Porrero MC, Mentaberre G, *et al.*, 2013. Antimicrobial resistance in indicator *Escherichia coli* isolates from free-ranging livestock and sympatric wild ungulates in a natural environment (Northeastern Spain). Appl Environ Microbiol 79:6184-6.

Semedo-Lemsaddek T, Nobrega CS, Ribeiro T, *et al*., 2013. Virulence traits and antibiotic resistance among *Enterococci* isolated from Eurasian otter (*Lutra lutra*). Vet Microbiol 163:378-82.

Seputiene V, Povilonis J, Ruzauskas M, *et al.,* 2010. Prevalence of trimethoprim resistance genes in *Escherichia coli* isolates of human and animal origin in Lithuania J Med Microbiol 59:315-22.

Yu-xi Z, Xian-Gan H, Jia-Kun Z, *et al*., 2015. Distribution of lipopolysaccharide core type in avian pathogenic *Escherichia coli* and its correlation with virulence gene. Microbiol China 42:1619-25.

Zhang AY, Wang HN, Tian GB, *et al.*, 2009. Phenotypic and genotypic characterization of antimicrobial resistance in faecal bacteria from 30 Giant pandas. Int J Antimicrob Agents 33:456-60.

Zhang C, Liu Y and Wang J, 2010. Detection of ESBLs and antimicrobial susceptibility of *Escherichia coli* isolated in Henan, China. J Anim Vet Adv 9:2030-4.

Zhang H, Rehman MU, Li K, *et al*., 2017. Antimicrobial resistance of *Escherichia coli* isolated from Tibetan piglets suffering from white score diarrhea. Pak Vet J 37:43-6.